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Expression, Self-Assembly, and Antigenicity of the Norwalk Virus Capsid Protein

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Norwalk virus capsid protein was produced by expression of the second and third open reading frames of the Norwalk virus genome, using a cell-free translation system and baculovirus recombinants. Analysis of the expressed products showed that the second open reading frame encodes a protein with an apparent molecular weight of 58,000 (58K protein) and that this protein self-assembles to form empty viruslike particles similar to native capsids in size and appearance. The antigenicity of these particles was demonstrated by immunoprecipitation and enzyme-linked immunosorbent assays of paired serum samples from volunteers who developed illness following Norwalk virus challenge. These particles also induced high levels of Norwalk virus-specific serum antibody in laboratory animals following parenteral inoculation. A minor 34K protein was also found in infected insect cells. Amino acid sequence analysis of the N terminus of the 34K protein indicated that the 34K protein was a cleavage product of the 58K protein. The availability of large amounts of recombinant Norwalk virus particles will allow the development of rapid, sensitive, and reliable tests for the diagnosis of Norwalk virus infection as well as the implementation of structural studies.

Norwalk and Norwalk-like viruses are important human pathogens that cause epidemic acute gastroenteritis (1, 16). Viruses in this group are spread by the fecal-oral route, and outbreaks of waterborne and food-borne gastroenteritis are well documented. Previous studies have estimated that at least 42% of outbreaks of nonbacterial gastroenteritis in the United States are caused by Norwalk or Norwalk-like viruses (18).

Although the 27-nm-diameter Norwalk virus was discovered almost 20 years ago, limited information has been available on the molecular characteristics of the virus because the virus has not been propagated in any cell culture system or a suitable, readily available laboratory animal model (16). Norwalk virus was found to induce infection, but not illness, in the chimpanzee model. Susceptible chimpanzees developed an antibody response, as detected by immunoelectron microscopy and radioimmunoassay, and in addition, certain animals shed an antigen detected by radioimmunoassay (30). As a result, the only source of Norwalk virus particles has been human stools, which characteristically contain very low concentrations of virus. Until now, the available immunologic diagnostic tests have included immunoelectron microscopy, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and Western immunoblotting, all of which require the use of reagents such as serum and stool samples from infected volunteers (8–10, 17). These assays were important in elucidating the natural history and basic characteristics of Norwalk virus and Norwalk virus infection. However, these assays were of limited use because of relatively poor test sensitivity, lack of sufficient antigen to enable large-scale epidemiologic or extensive laboratory studies, and possible variability of results due to the use of samples from different volunteers.

Previous information on the molecular characterization of Norwalk virus indicated that the viral capsid contained a single protein with a molecular weight of approximately 60,000 (60K protein) (7). In studies of other viruses with similar properties (Snow Mountain agent; small, round-structured viruses; and human caliciviruses), others found the capsid contained a single protein, the 62K or 63K protein (21, 27). For Norwalk virus, a 59K protein and a soluble 30K protein were identified as antigens that reacted with some human convalescent-phase serum samples following immunoprecipitation of antigen partially purified from stools and radiolabeled with iodine (7). Similar proteins (63K and 33K proteins) of a small, round-structured virus isolated in Japan also reacted by Western blotting (9).

Recently, we cloned the Norwalk virus genome (14). Three open reading frames (ORFs) were identified in the Norwalk virus genome on the basis of analysis of the nucleotide sequences and predicted amino acid sequences (14). This genomic organization is consistent with the Norwalk virus being classified as a member of the *Caliciviridae* (14). The second ORF was predicted to encode a 56.6K protein with approximately the same apparent molecular weight as the Norwalk virus capsid protein. This study shows that the capsid protein is encoded in the second ORF by expression studies using a cell-free transcription-translation system and baculovirus recombinants. In addition, this recombinant protein self-assembles into viruslike particles that are antigenic and immunogenic.

MATERIALS AND METHODS

Subcloning of Norwalk virus clones into the pGEM transcription vector and a baculovirus transfer vector. The predicted genomic organization of Norwalk virus and the generation of clone pUCNV-4145 are described in detail by Jiang et al. (13, 14). In this study, subgenomic cDNA fragments of the Norwalk virus genome were subcloned into plasmid vector pGEM-7zf(+) (Promega, Madison, Wis.) and used for in vitro transcription and translation. pGNV-2 was a pGEM subclone of the *Hin*dIII fragment of pUCNV-4145 (13) and contained the entire second ORF of the Norwalk virus genome (Fig. 1). pGNV-d2-3 contained part of the

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second ORF, lacked the first two of three in-phase AUG initiation codons, and contained the entire third ORF of the Norwalk virus genome (Fig. 1). The baculovirus transfer vector pVL1393 was used for subcloning of the viral cDNA. pVLNV-2-3 contained the partially *Eco*RI-digested 2.4-kb fragment of the 3′ end of the Norwalk virus genome inserted into the *Eco*RI site of the baculovirus transfer vector pVL1393 (29) (Fig. 1). This clone contained the entire second and third ORFs of the Norwalk virus genome.

In vitro transcription and translation. Plasmid DNAs pGNV-2 and pGNV-d2-3 were linearized by restriction enzyme digestion of the 3′ end of the inserted DNA. RNA transcripts from these linearized DNAs were made by in vitro transcription with SP6 RNA polymerase by using the conditions recommended by the manufacturer (GIBCO BRL, Gaithersburg, Md.). Norwalk virus-specific proteins were produced and labeled with [^{35}S]methionine (1,056 Ci/mmol) by in vitro translation of the synthetic RNA transcripts using rabbit reticulocyte lysates (prepared in this laboratory) as described previously (22).

Production of baculovirus recombinants. Insect (*Spodoptera frugiperda*) cells (Sf9) were cotransfected with wild-type baculovirus DNA and plasmid pVLNV-2-3 DNA as previously described (5). Recombinant viruses were identified by dot blot hybridization followed by three rounds of plaque purification. Expressed viral proteins were analyzed following infection of Sf9 cells with plaque-purified virus at a high multiplicity of infection (~10) and harvesting of the cells 4 to 5 days postinfection. The supernatant, which contained most of the expressed capsid protein, was separated from the cell lysate by centrifugation of the culture for 15 min at 3,000 rpm in a Beckman JA-17 rotor. The expressed proteins in the supernatant were concentrated either by precipitation with polyethylene glycol (8%) or centrifugation through a sucrose cushion (see below) before being analyzed by electrophoresis in 10% polyacrylamide gels. The proteins were analyzed in the presence of sodium dodecyl sulfate and 2-mercaptoethanol with or without precipitation, with preinfection or 28-day-postinfection serum samples obtained from volunteers given Norwalk virus. Proteins were analyzed by fluorography or stained with Coomassie blue or silver nitrate as previously described (22).

Purification of the 58K capsid protein. Supernatants of recombinant baculovirus-infected cell cultures were extracted once with trichlorotrifluoroethane (genetron). The viral protein was then concentrated by centrifugation through a 40% sucrose cushion for 2 h at 35,000 rpm, using a Beckman 50.2 Ti rotor. The resultant pellets were suspended in a solution of CsCl (1.362 g/cm^3) and centrifuged at 35,000 rpm for 24 h in a Beckman SW 50.1 rotor. The 58K protein was identified by electrophoretic analysis of aliquots of the fractions from the gradient. The peak fractions of 58K protein were pooled and pelleted by centrifugation for 2 h at 35,000 rpm in a Beckman SW 50.1 rotor to remove the CsCl. Fractions containing the purified protein were examined by negative-stain electron microscopy (EM) and used as antigen to immunize animals (see below).

N-terminal amino acid sequencing of the 58K and 34K proteins. The 58K and 34K proteins purified from the insect cell supernatants and cell lysates, respectively, were electrophoresed in polyacrylamide gels without urea. The proteins in the gels were transferred onto Immobilon-P transfer membranes (Millipore, Bedford, Mass.) and stained with Coomassie blue. The bands containing the two proteins were cut out and sequenced on an Applied Biosystems 477A protein sequencer in the Baylor College of Medicine Protein Chemistry Core Facility.

Production of hyperimmune antiserum in laboratory animals. To produce hyperimmune serum, mice, guinea pigs and rabbits were immunized with the 58K recombinant Norwalk virus (rNV) capsid protein. The immunization regimen consisted of one intramuscular injection of the purified rNV in Freund's complete adjuvant (with a dose of 80 μg per mouse, 200 μg per guinea pig, and 300 μg per rabbit) followed by two booster injections of the same dose in Freund's incomplete adjuvant. The animals were bled 2 weeks after the last booster injection.

EM. Direct EM was performed with negative staining of the samples with 1% ammonium molybdate. Stability of the viruslike particles was determined by EM examination after exposure of virus preparations to different pHs and temperatures and to lyophilization.

ELISA to detect Norwalk virus antibody in serum using rNV capsid protein as antigen. An ELISA was performed using a modified version of the format developed to detect rotavirus (3). Purified rNV capsid protein was used as antigen to coat polyvinylchloride microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) at a concentration of 1 μg/ml (100 μl per well), and the plate was incubated for 4 h at 37°C. The antigen-coated microtiter plate was then blocked with 5% Blotto (Carnation nonfat milk) in 0.01 M phosphate-buffered saline (PBS) overnight at 4°C. After each well was washed two times (200 μl per well) with 0.05% Tween 20–PBS (TW-PBS), serial dilutions of human serum samples (100 μl per well diluted in 1% Blotto–PBS) were added to the wells and the plate was incubated for 2 h at 37°C. After each well was washed six times with TW-PBS, the bound antibody was detected by adding horseradish peroxidase-conjugated goat anti-human immunoglobulin G [IgG], IgA, and IgM (Cappel Organon Teknika Corp., West Chester, Pa.) (1:5,000 dilution in 1% Blotto–PBS; 100 μl per well) and the plate was incubated for 2 h at 37°C. After each well was washed six times with TW-PBS, the substrate 2,2′-azinobis(3-ethylbenz-thiazoline sulfonic acid) (ABTS; Sigma Chemical Co., St. Louis, Mo.) was added (100 μl per well). The plate was kept at room temperature for 30 min before being read at A_{414}. Test samples were considered positive if they showed an A_{414} reading of ≥0.1 on the basis of test results obtained with known positive and negative serum samples (12).

RESULTS

The predicted second ORF in the Norwalk virus genome encodes a 58K protein. The Norwalk virus genome is predicted to encode three ORFs (Fig. 1) (14). The first ORF, located at the 5′ end of the viral genome, is predicted to encode a protein of 1,739 amino acids (aa), with a molecular weight of 193,000. The second ORF, which follows the first ORF (with a few bases of overlap), is predicted to encode a 531-aa protein with a molecular weight of 56,600. The third ORF, located at the 3′ end of the genome, should encode a 213-aa or 22.5K protein. We hypothesized that the second ORF might encode the viral capsid protein because the predicted size of this protein was similar to that of the Norwalk viral capsid protein reported previously (7). To confirm this hypothesis, we examined the proteins made following in vitro transcription and translation of the Norwalk virus cDNAs using rabbit reticulocyte lysates. The translation products of clones pGNV-2 and pGNV-d2-3 were analyzed by gel electrophoresis after immunoprecipitation

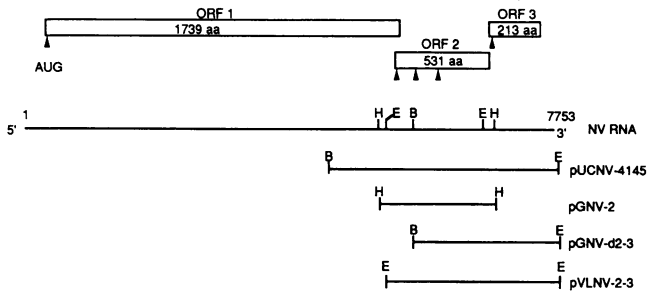

FIG. 1. Subcloning of the second and third ORFs of the Norwalk virus genome into pGEM transcription vectors and a baculovirus transfer vector. pGNV was a subclone of the *Hin*dIII fragment of pUCNV-4145 inserted into the pGEM vector. This subclone contained the entire second ORF of the Norwalk virus genome and a small part (59 aa) of the third ORF. pGNV-d2-3 contained the entire third ORF and part of the second ORF (the first two strong AUG initiation codons [▲] were missing in this construct). To obtain baculovirus recombinants, the pUCNV-4145 clone containing the second and third ORFs of the Norwalk virus genome was partially digested with *Eco*RI. The 2.4-kb fragment covering both ORFs was isolated by agarose gel electrophoresis. The recovered DNA fragment was then ligated into the baculovirus transfer vector pVL1393 at the *Eco*RI site. The orientation of the fragment in the vector was determined by restriction enzyme and sequence analyses. Clones containing the correct orientation of the Norwalk virus cDNA insert in the transfer vector (the viral DNA was under the control of the polyhedrin gene of baculovirus) were selected for further experiments to produce baculovirus recombinants. The region of RNA-dependent RNA polymerase described previously (13) is located at the 3′ end of ORF 1. The GenBank accession number of the Norwalk virus sequence is M87661. Abbreviations: NV, Norwalk virus; H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI.

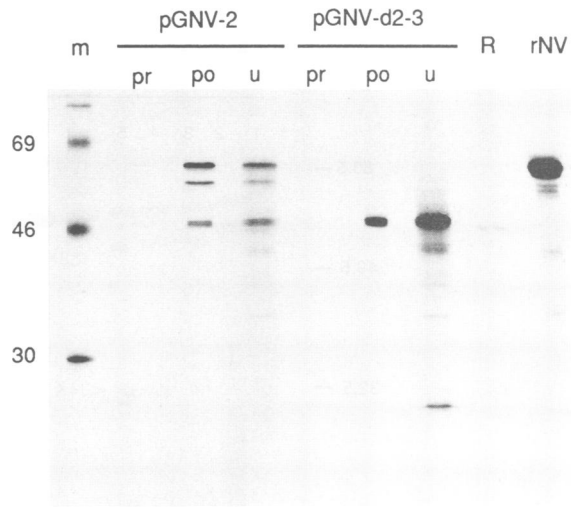

FIG. 2. In vitro transcription and translation of the Norwalk virus subclones. In vitro transcription was performed by using SP6 RNA polymerase. The transcribed RNAs were then translated and labeled with [^{35}S]methionine, using rabbit reticulocyte lysates. The translated proteins were analyzed by electrophoresis on a 10% polyacrylamide gel either directly (u) or after immunoprecipitation with a serum sample taken preinfection (pr) or postinfection (po) from a volunteer infected with Norwalk virus. The positions (in thousands) of molecular weight markers (lane m) of ^{14}C-methylated proteins from Amersham, Arlington Heights, Ill., are shown to the left of the gel. R, rabbit reticulocyte lysate translation products made without any exogenous RNA added. rNV, Norwalk virus capsid protein expressed in baculovirus recombinant. (see below).

with serum samples collected from volunteers before or after infection with Norwalk virus. pGNV-2 contained a *Hin*dIII fragment of pUCNV-4145, and this fragment covered the entire second ORF of the Norwalk virus genome. Translation of transcripts from pGNV-2 revealed three bands in the gel with apparent molecular weights of 58,000, 50,000, and 46,000, respectively (Fig. 2). All three proteins were precipitated by postinfection, but not preinfection, serum samples from infected volunteers. We hypothesized that the largest band was the full-sized viral capsid protein and that the two smaller proteins were derived from two internal initiation codons present in the second ORF. This hypothesis was confirmed when the translated products of pGNV-d2-3 were assayed. pGNV-d2-3 contained only a portion of the second ORF (starting from the third AUG). This construct directed the synthesis of only one protein that comigrated with the smallest protein made from pGNV cDNA. pGNV-d2-3 also contained the predicted third ORF of the Norwalk virus genome. However, a protein of the predicted size for the third ORF was not observed in the gel.

Production of the Norwalk virus capsid protein in the baculovirus expression system. We also subcloned a 2.4-kb partially *Eco*RI-digested fragment of pUCNV-4145 into a baculovirus transfer vector to produce a construct called pVLNV-2-3 (Fig. 1). After cotransfection with wild-type baculovirus DNA, selection, and plaque purification of recombinant virus, insect cells were infected with isolated stocks (called C-6 and C-8) of baculovirus recombinants containing Norwalk virus cDNA. Electrophoretic analysis of the expressed proteins after electrophoresis in a polyacrylamide gel and staining with Coomassie blue showed a major band with an apparent molecular weight of 58,000. This was seen only in rNV-infected cells, not in wild-type baculovirus- or in mock-infected cells (data not shown). Kinetic experiments showed that this protein was released into the medium of the culture from the third day until at least the fifth day postinfection (Fig. 3). A minor band with a molecular weight of about 34,000 was also seen in the same infected-cell cultures, but the 34K protein was mainly cell associated (Fig. 3 and 4). To determine the origin of the 34K protein, amino acid sequencing of the 34K and 58K proteins was performed. The 58K protein was blocked. The first 10 aa of the amino terminus of the 34K protein were identical to the 10 aa between aa 217 and 226 of the 58K protein. Therefore, the 34K protein apparently was a cleavage product of the 58K protein. The small protein remained immunoreactive after cleavage, as it was immunoprecipitable by convalescent-phase serum samples from an infected volunteer (Fig. 4).

The 58K protein forms viruslike particles. Because the 58K protein was released into the culture medium, the procedure for purifying the protein was greatly simplified. Figure 5 shows the electrophoretic analysis of the 58K protein purified from the supernatant of infected cells after centrifugation in a CsCl gradient. A major peak of 58K protein was seen at a density of 1.31 g/cm^3. Viruslike particles were observed when the peak fractions were examined by EM (Fig. 6). These rNV particles were morphologically similar to the authentic viral particles purified from human stools. The rNV appeared empty by EM, suggesting that they lacked nucleic acid. This was confirmed by the subsequent lack of hybridization of the purified particles with Norwalk virus-specific DNA probes (data not shown). The rNV

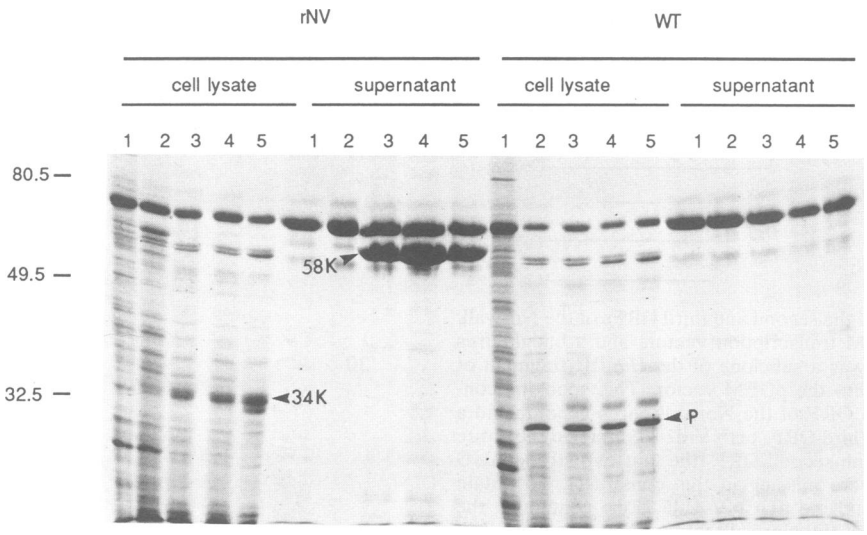

FIG. 3. The Norwalk virus capsid protein is released into the medium of infected insect cells. Insect cells were infected with recombinant C-8 (rNV) or wild-type baculovirus (WT) at a high multiplicity of infection (~10), and the infected cells were harvested at different days postinfection. The supernatant of the culture was separated from the cells by low-speed centrifugation (15 min at 3,000 rpm in a Beckman JA-17 rotor). The expressed viral proteins in the supernatant were concentrated by precipitation with 8% polyethylene glycol followed by centrifugation for 30 min at 15,000 × *g*. The resultant pellets were dissolved in water and adjusted to a volume equal to that of the cell lysates. Aliquots of the same amounts of cell lysate and supernatant were electrophoresed on a 10% polyacrylamide gel that was stained with Coomassie blue. The day of harvesting (days 1 to 5) after infection is indicated above each lane. The positions (in thousands) of molecular weight markers are shown to the left of the gel. P, baculovirus polyhedrin.

particles were stable for at least 6 months stored at both 4 and −20°C and were stable after lyophilization. The particles were resistant to acid treatment (pH 3.0 for 10 min), but were not stable to exposure to pH 10 for 10 min. Yields of 6.5 to 12.5 mg of purified rNV were routinely obtained from 100-ml samples of recombinant virus-infected insect cell cultures.

The rNV particles are antigenic. The antigenicity of the 58K protein was shown by its specific reaction by immunoprecipitation with paired serum samples from a volunteer challenged with Norwalk virus (Fig. 4). To further confirm these results, the rNV was used as coating antigen in an ELISA to detect Norwalk virus-specific antibody in paired serum samples obtained from four volunteers challenged with Norwalk virus. Significant increases of reactivity were observed in the postchallenge serum samples compared with that of prechallenge serum samples from volunteers infected with Norwalk virus (Fig. 7). Reconstituted lyophilized particles also retained their antigenicity.

The rNV particles are immunogenic. CsCl gradient-purified rNV was used to immunize mice, guinea pigs, and rabbits. After three injections of rNV, all three animal species produced serum samples with high titers of antibodies against rNV ($\geq 1{:}10^6$) by the recently developed ELISA. In addition, these animals also developed high levels of antibodies to the authentic Norwalk virus particles obtained from volunteers (1:256,000 for rabbit serum, $>1{:}10^6$ for guinea pig serum, and 1:256,000 for mouse serum). Preliminary experiments indicated that the ELISA using these serum samples was highly specific and more sensitive than the previously described radioimmunoassay and enzyme immunoassay using human stool and serum samples for the detection of Norwalk virus infection (data not shown) (12). Development and evaluation of diagnostic tests using these hyperimmune sera for Norwalk virus infection are in progress.

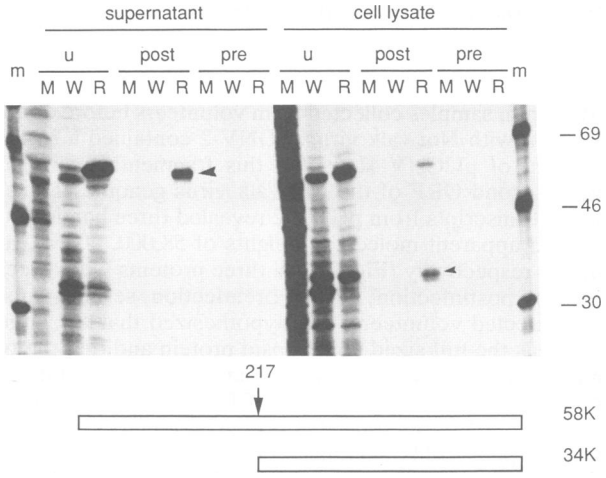

FIG. 4. Two related proteins, the 58K and 34K proteins, are present in infected insect cells. Insect cells were infected with the baculovirus recombinant C-8 in the presence of [^{35}S]methionine and harvested at 5 days postinfection. Proteins in the supernatant and cell lysate immunoprecipitated with pre- or postinfection serum samples from a volunteer infected with Norwalk virus ([pre] or [post]) or not immunoprecipitated (u) were analyzed by gel electrophoresis in a 10% polyacrylamide gel. Lanes: M, mock infection; W, insect cells infected with wild-type baculovirus; R, insect cells infected with baculovirus recombinant C-8; m, molecular weight markers, as described in the legend to Fig. 2. The positions (in thousands) of molecular weight markers are shown to the right of the gel. The large arrowhead points to the 58K protein, and the small arrowhead points to the 34K protein. The bottom panel shows the potential relationship between the 58K and 34K proteins. The amino acid sequence of the 34K protein matched the 10 aa of the 58K protein between aa 217 to 226, suggesting that the 34K protein was a cleavage product of the 58K protein.

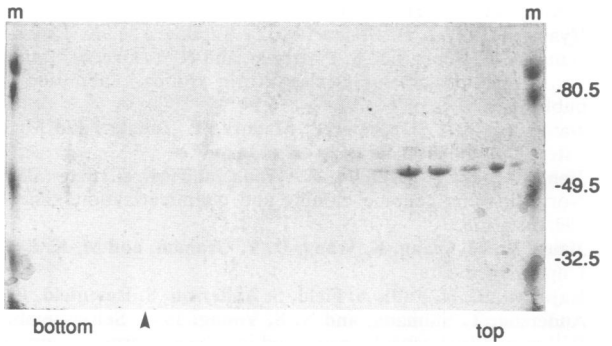

FIG. 5. Norwalk virus capsid protein purified by centrifugation in CsCl gradients. The supernatant of the baculovirus recombinant-infected insect cells was extracted with genetron followed by precipitation with polyethylene glycol (8%). Virus in the polyethylene glycol pellet was suspended in water, then mixed with a solution of CsCl (final density of 1.362 g/cm^3), and centrifuged for 24 h at 35,000 rpm in an SW 50.1 rotor. Aliquots from the gradient were analyzed by electrophoresis on a 10% polyacrylamide gel and stained with Coomassie blue. The bottom and top of the gradient and the positions (in thousands) of molecular weight markers (m lanes) are shown. The fraction (density of 1.38 g/cm^3 in CsCl) expected to contain complete full viral particles is indicated (▲). The density in CsCl of the expressed viral particles was ~1.31 g/cm^3.

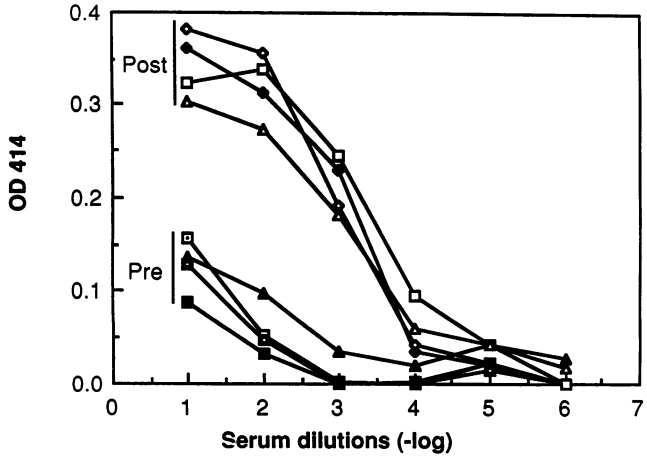

FIG. 7. Immunoreactivity of recombinant Norwalk virus particles with the pre- and postchallenge serum samples from four volunteers infected with Norwalk virus. Purified rNV particles were used to coat the wells of microtiter plates. Serial dilutions of serum samples from volunteers infected with Norwalk virus were added to each well, and the bound antibodies were detected by horseradish peroxidase-conjugated goat anti-human IgG, IgM, and IgA. Substrate (ABTS) was added, and the optical density at 414 nm (OD 414) after 30-min development was read. Pre and Post, serum samples collected from volunteers before and 21 to 35 days after challenge, respectively.

DISCUSSION

Synthesis of foreign proteins in the baculovirus expression system has been proven to be an excellent technique for producing large quantities of protein (29). Proteins produced using the baculovirus expression system usually preserve the biologic properties of their native counterparts, and recently, this system has been shown to be able to produce self-assembled viruslike particles for both RNA and DNA virus systems (2, 15, 19, 20, 26, 28).

By expression of one-third of the 3′ end of the Norwalk virus genome, we have demonstrated several key features about this virus. First, we have demonstrated that the second predicted ORF of the Norwalk virus genome encodes the viral capsid protein. This protein with an apparent molecular weight of 58,000 could also self-form into viruslike particles and these particles are immunoreactive (Fig. 6 and 7). This accomplishment now provides a virtually unlimited supply of highly purified viral capsids that has allowed the development of sensitive and specific tests for Norwalk virus detection. The usefulness of rNV to detect serologic responses has been confirmed by others (6, 11). The simple procedure described here for the purification of the protein, together with the stability of the protein to lyophilization and acid treatment, suggests that this rNV could also be a vaccine candidate.

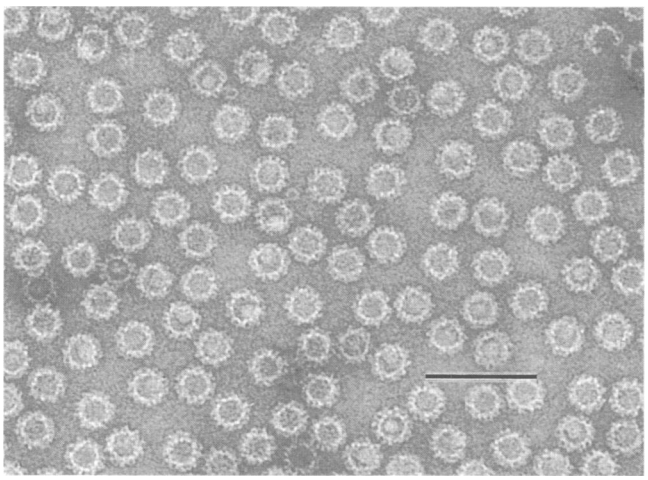

FIG. 6. Electron micrograph of purified Norwalk virus capsid protein from infected insect cells. Virus obtained from a CsCl gradient was diluted 1:100 in distilled water, and the samples were stained with 1% ammonium molybdate, pH 6.0. Homogeneous viruslike particles were seen in every grid square. Bar, 100 nm.

Second, it is clear that expression of the second ORF produced not only a 58K protein but also a 34K protein. These results are consistent with previous reports that the capsid protein (59K protein) and a soluble antigen of approximately 30K are present in the stools of volunteers infected with Norwalk virus (7). However, our present data cannot determine whether the production and relationship between the 58K and 34K proteins seen in insect cells infected with the baculovirus recombinant are similar to those in the human intestine. The 34K protein was not present in the rNV particles described above. Therefore, the biologic role of the protein in the virus replication cycle remains to be determined. The fact that the 34K protein was immunoprecipitated by convalescent-phase serum samples indicates the carboxy terminus of the capsid protein contains epitopes.

The third ORF of the Norwalk virus genome was not expressed or not efficiently expressed in either the in vitro translation system or the baculovirus expression system. Whether this ORF is expressed during virus infection and what function this protein may play in the virus replication cycle are still not clear. The expression and translation of a subgenomic RNA have been clearly proven for other caliciviruses, such as feline calicivirus and rabbit hemorrhagic disease virus (4, 23–25). Subcloning of an optimal-sized

cDNA for making RNA covering only the third ORF may be required for expression of this third ORF protein.

Preliminary results have indicated that immunologic tests using rNV are quite specific for Norwalk virus (Fig. 7; also data not shown), and there has been little cross-reactivity in detecting serologic responses in volunteers given viruses of the Norwalk virus-like group (6, 12). It remains unknown whether development of immunologic tests using nonstructural proteins as the viral antigen will be a useful supplement for the tests described here that use the expressed structural protein.

Another potential application of the expressed viral capsid protein is to study cell-virus interactions in binding experiments. This should allow us to screen large numbers of cell lines for specific binding. By doing this screening, it may be possible to identify specific cell lines suitable for the growth of Norwalk virus. Finally, another application of the viral capsid protein may be the in vitro assembly of particles with Norwalk virus-specific RNA derived from in vitro transcription. This may generate infectious virus-like particles and eventually permit virus growth in cell culture.

ACKNOWLEDGMENTS

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